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Role of Tetraspanins CD9 and CD151 in Primary Melanocyte Motility

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Tetraspanins CD9 and CD151 have been implicated in cellular motility and intercellular adhesion in several cellular types. Here, we have studied the subcellular localization and the functional role of these molecules in primary melanocytes. We found that endogenous tetraspanins preferentially clustered in areas of melanocyte homotypic intercellular contacts and at the tips of dendrites. These observations were further confirmed using time-lapse fluorescence confocal microscopy of melanocytes transfected with CD9– and CD151–GFP (green fluorescent protein) constructs, suggesting an involvement of these proteins in cellular contacts and migration. Cell adhesion and migration assays performed using blocking monoclonal antibodies against CD9 and CD151 showed no significant effect on cell–extracellular matrix adhesion, whereas the migration of melanocytes was significantly enhanced. The regulation of the migratory capacity of melanocytes by CD9 and CD151 was further confirmed knocking down the endogenous expression of these tetraspanins with small interference RNA oligonucleotides. Therefore, tetraspanin molecules are localized at motile structures in primary human melanocytes regulating the migratory capacity of these cells.

Key words: CD151/CD9/cell adhesion/cell migration/intercellular contacts/melanocytes
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Tetraspanins comprise a numerous group of proteins that contain four putative membrane-spanning domains and, characteristically, the presence of a large divergent extracellular loop between the third and fourth membrane-spanning domains (Wright and Tomlinson, 1994). They have been implicated in the regulation of cell development, proliferation, activation, and motility and have been shown to couple to signal transduction pathways (Berditchevski, 2001; Boucheix and Rubinstein, 2001; Hemler, 2001; Stipp *et al*, 2003). In this regard, it has been suggested that the main role of tetraspanins is to organize other proteins into signal-transducing complexes at the cell surface (Berditchevski, 2001; Yáñez-Mó *et al*, 2001; Tarrant *et al*, 2003). Tetraspanins have been shown to coprecipitate with several transmembrane proteins, and with β1 integrins in particular (Berditchevski *et al*, 1996; Hemler *et al*, 1996; Rubinstein *et al*, 1996; Maecker *et al*, 1997). In epidermal cell adhesion and migration, β1 integrins play a critical role (Watt and Hertle, 1994). To participate in those functions, integrins may not only bind to intracellular proteins and extracellular ligands (Clark and Brugge, 1995) but may also laterally associate with other transmembrane proteins such as those from the tetraspanin superfamily (Berditchevski, 2001; Boucheix and Rubinstein, 2001; Hemler, 2001; Stipp *et al*, 2003).

The migration of melanocytes represents a fundamental requirement in a wide variety of physiological and pathological scenarios. Melanocytes migrate from the neural crest to the skin during the first trimester of the embryogenesis. In physiological conditions, melanocytes migrate to display a homogeneous distribution in the epidermis, which have been designated as an "epidermal melanin unit" (Fitzpatrick and Breathnach, 1963). But insights into the cellular and molecular mechanisms underlying such organization, however, remain unknown (Hoath and Leahy, 2003). In adults, melanocytes also migrate during the process of wound healing and to cover the white areas of patients with vitiligo. Although integrins (Morelli *et al*, 1993) and cadherins (Hsu *et al*, 2000) are both implicated in adhesion and movement of melanocytes, little is known about other factors that regulate both functions. Tetraspanins have been found on normal epidermis (Okochi *et al*, 1997; Sincock *et al*, 1997), expressed by keratinocytes (Jones *et al*, 1996; Okochi *et al*, 1997; Peñas *et al*, 2000). We have previously demonstrated that tetraspanin molecules have an important role in keratinocyte motility (Peñas *et al*, 2000). On the other hand, CD9 has been functionally associated with tumor cell motility (Ikeyama *et al*, 1993) and melanoma invasion (Longo *et al*, 2001). Nevertheless, no formal study of tetraspanins has been performed in human primary melanocytes. In this study, we describe the subcellular localization of CD9 and CD151 tetraspanin molecules and their functional role in melanocyte motility.

Abbreviations: ECM, extracellular matrix; GFP, green fluorescent protein; mAb, monoclonal antibody; siRNA, small interference RNA

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Results

Melanocytes express CD9 and CD151 tetraspanin proteins at intercellular contacts and tips of dendrites We analyzed the expression of tetraspanins CD9, CD63, CD81, and CD151, and integrins αvβ3, β1, α2, and α3 by flow cytometry in normal human melanocytes. These primary cells express high levels of β1 integrin and CD9, and lower levels of α2 and α3 chains of integrins and tetraspanins CD81, CD151, and CD63 (Fig 1).

Immunofluorescence studies showed that CD9 and CD151 are distributed throughout the cell body and the dendrites of the melanocyte, their clustering at points of intercellular contact and at the tips of dendrites being remarkable, where they preferentially colocalized with β1 integrins (Fig 2*a* and *b*). The colocalization percentage obtained considering the overall surface of the melanocytes, however, is rather average (ranging from 14% to 28%), and not all contacts and tips contained accumulation of both molecular types (data not shown). CD81 exhibits a staining pattern similar to that of CD9 and CD151, but no clear CD63 membrane expression was found, although it was highly represented in intracellular vesicles as it has been found in other cell types such as endothelial cells and keratinocytes (data not shown) (Yáñez-Mó *et al*, 1998; Peñas *et al*, 2000).

We found footprints in the immunofluorescence studies with CD9 but almost no staining with CD151 (data not shown). These footprints are made of rests of plasma membrane containing receptors for extracellular matrix proteins that remain attached to the ligands as the cell moves. We and other authors have described this phenomenon, also termed ripping, in keratinocytes (Peñas *et al*, 2000) and other cell types (Palecek *et al*, 1996).

As these findings suggested an implication of tetraspanins in adhesion to the extracellular matrix, we performed adhesion experiments on collagen. We used TS2/16 (an anti-CD29 that activates integrins β1) and VJ1/14 (an anti-CD29 that inhibits integrins β1) monoclonal antibodies (mAb) as positive and negative controls of adhesion, respectively. Differences between both TS2/16 (enhancing adhesion) and VJ1/14 (inhibiting adhesion) were significant ($p < 0.005$) but moderate ($\pm$ 15% from the control). When melanocytes were incubated with anti-tetraspanin mAb, we also found a reproducible but very mild modification of adhesion ($\leqslant$15%) compared with control cells without antibody treatment, suggesting the lack of implication of CD9 and CD151 in adhesion to collagen (data not shown) as described for other cell types (Yáñez-Mó *et al*, 1998).

Tetraspanins CD9 and CD151 are involved in melanocyte–melanocyte interactions To ascertain the kinetics of the localization of tetraspanins in intercellular homotypic contacts of melanocytes, we erformed time-lapse fluorescence confocal microscopy with CD9–GFP (green fluorescent protein) and CD151–GFP-tagged proteins. Using CD9–GFP we found that this molecule showed a diffuse pattern of accumulation at transient cell–cell contact sites (Fig 3*a*, Fig S1). CD151–GFP was rapidly and highly concentrated as soon as dendrites of different melanocytes come into contact (Fig 3*b*, Fig S2). Both findings

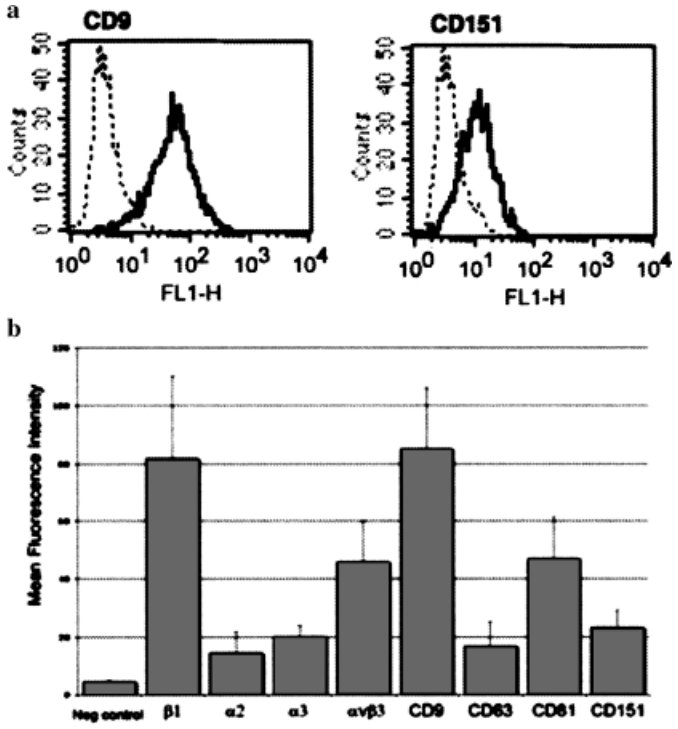

Figure 1
Human primary melanocytes express tetraspanins CD9, CD63, CD81, and CD151. (*a*) Representative profiles of flow cytometry analysis corresponding to CD9 and CD151 tetraspanins in melanocytes. (*b*) A quantitative analysis of the membrane expression of β1, α2, α3, and αvβ3 integrins and CD9, CD63, CD81, and CD151 tetraspanin proteins expressed as mean fluorescence intensity measured by flow cytometry (mean + SEM of six to nine experiments). X-63 was used as negative control.

suggest that CD9 and CD151 transiently cluster at the homotypic intercellular contacts during the scanning of the melanocyte cell surface.

Tetraspanins are implicated in melanocyte motility On the other hand, another specific area of accumulation was the tips of the dendrites, which are enriched in ruffles. We found enhanced expression of CD9–GFP (Fig 4*a*, Figure S3) and CD151–GFP (Fig 4*b*, Fig S4) at the tip of dendrites when melanocytes explore the surrounding milieu. This localization was dynamic and was displayed by all dendrites; those that directed the movement of the melanocyte and those that were retracting.

As these results suggested that CD9 and CD151 may be involved in the movement of melanocytes, they prompted us to formally explore the functional involvement of tetraspanins in melanocyte motility using Transwell chambers with fibronectin in the lower chamber as a stimulus for melanocyte motility. Blocking monoclonal antibodies against CD9 and CD151, as determined in other cellular types (Yáñez-Mó *et al*, 1998; Peñas *et al*, 2000), enhanced melanocyte motility, whereas anti-αvβ3 integrin inhibited the migration of melanocytes. The activatory anti-β1 integrin TS2/16 decreased the migration of melanocytes, whereas the inhibitory anti-β1 integrin VJ1/14 increased it (Fig 5*a*). To evaluate the influence of β1 integrins in CD9 and CD151-mediated effect on melanocyte migration, we combined the use of TS2/16 and VJ1/14 with anti-CD9 and anti-CD151 (Fig 5*b*).

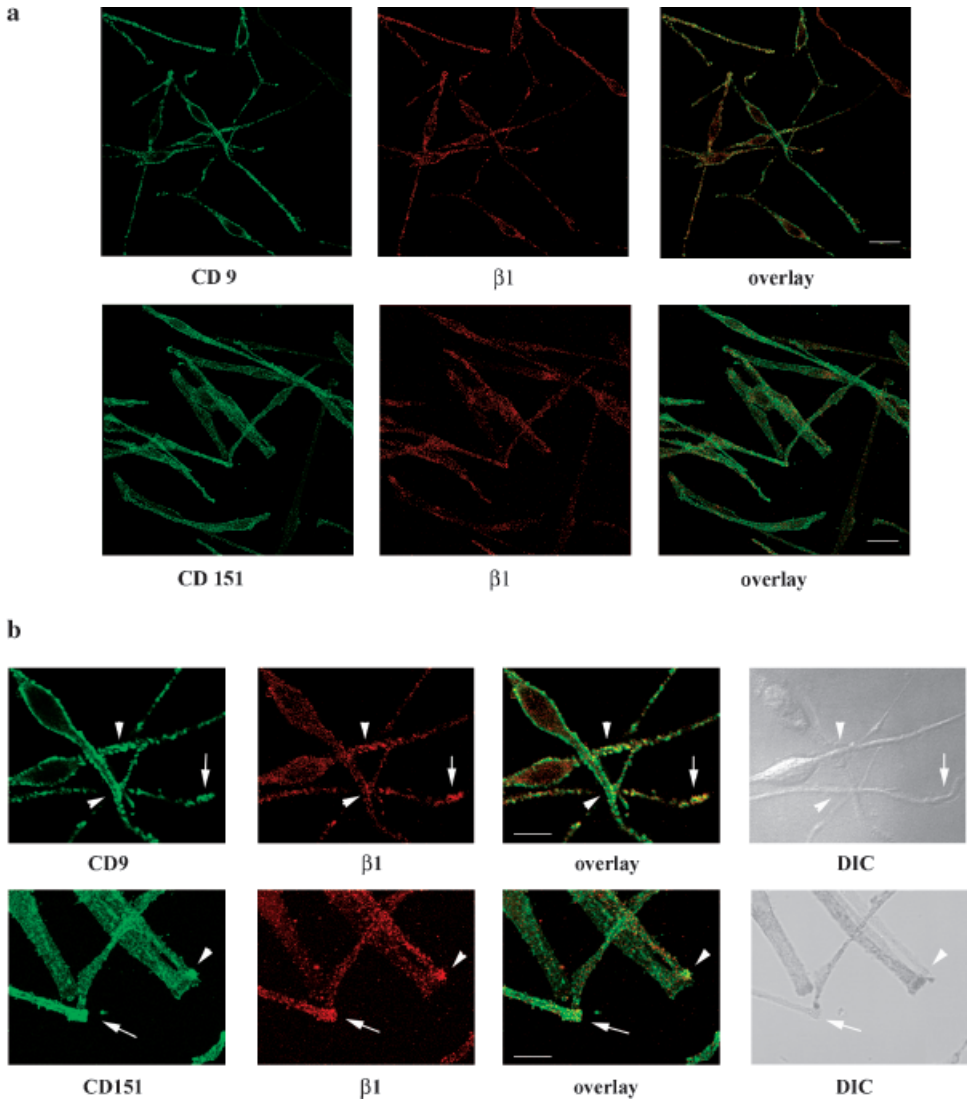

Figure 2
Tetraspanins and β1 integrins colocalize at intercellular contacts of melanocytes and at tips of dendrites. (*a*) Representative horizontal confocal sections showing the subcellular distribution of CD9 or CD151 (green fluorescence) compared with β1 integrin (red fluorescence), together with the overlay images are depicted. *Scale bar* = 20 μm. (*b*) Zoom images from the representative horizontal confocal sections shown in the previous panel are depicted. Co-clustering of tetraspanin proteins with β1 integrin at intercellular contacts are indicated with white arrowheads and at the tips of the dendrites are indicated with white arrows. *Scale bar* = 10 μm.

We found that the enhanced effect of CD9 and CD151 on melanocyte migration was more pronounced when combined with VJ1/14, and was partially reversed by TS2/16.

To further assess the functional role of tetraspanins in melanocyte motility, tetraspanin expression was knocked down in melanocytes using small interference RNA (siRNA) against CD9 and CD151. We obtained a significant inhibition in the expression of CD9 and CD151 using specific siRNA (Fig 6*a*), without affecting the morphological characteristics of siRNA-treated melanocytes (data not shown). We performed similar migration assays using fibronectin as a stimulus of melanocyte motility with these interfered cells and found that siRNA against CD9 and CD151 significantly ($p < 0.05$) stimulates melanocyte migration (Fig 6*b*). Altogether these data underscore the involvement of tetraspanin in melanocyte migration through regulation of β1 integrin function.

Discussion

The role of tetraspanins in keratinocytes has been explored previously (Peñas *et al*, 2000). Tetraspanins are implicated in keratinocyte motility, and a possible implication of these molecules in inter-keratinocyte contacts has been suggested. Although CD9, CD81, and CD151 have been described in melanoma cells (Radford *et al*, 1996; Longo *et al*, 2001), no description of the expression or functional implications of tetraspanins in normal human melanocytes has been reported before.

This work addresses the expression, subcellular distribution, and functional role in cell adhesion and motility of the tetraspanins CD9 and CD151 in primary human melanocytes. The accumulation of both tetraspanins at the tips of the melanocyte dendrites and in the intercellular contacts with other melanocytes was remarkable. Moreover, some ripping was found. These findings suggested the involvement of CD9 and CD151 in melanocyte adhesion to the extracellular matrix, in the exploration of the environment and in homotypic interactions. CD63, a component of melanosomes also known as lysosome-associated membrane protein (LAMP)-3, has been described in melanocytes and melanoma, and implicated in melanoma progression (Ota *et al*, 1998). As expected, CD63 showed low plasma membrane and high intracellular expression in primary melanocytes.

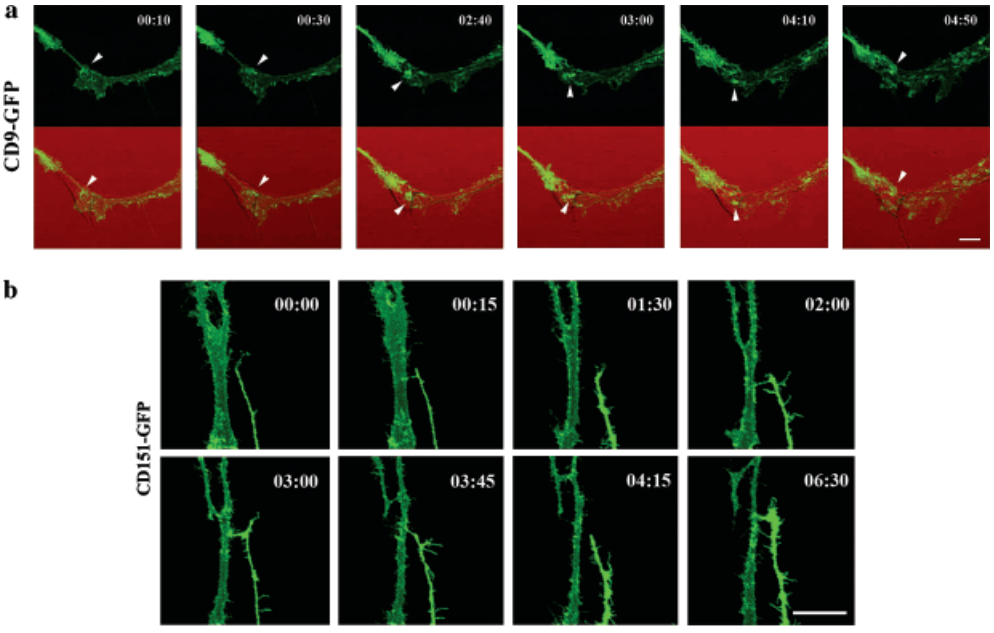

Figure 3
CD9—GFP (green fluorescent protein) and CD151–GFP are dynamically expressed in intercellular contacts. Melanocytes transfected with CD9–GFP or CD151–GFP constructs were seeded on coverslips and time-lapse fluorescence confocal microscopy was started after 4 h. Series of optical sections distanced 0.4 μm on the z-axis were acquired every 10 min (CD9) or 15 min (CD151) for a total time of 7 h. a) All the micrographs depicted show the transient accumulation of CD9–GFP at the tip of the dendrite when it contacts with another untransfected melanocyte (*white arrowheads*). A maximum projection of the relevant sections from the confocal stack of the GFP and the merged image also composed with the corresponding DIC image from each selected time point of the videosequence are shown. *Scale bar* = 10 μm. (*b*) The images show the accumulation of CD151–GFP on the membrane of the melanocyte as soon as the cell contacts the dendrite of another highly transfected melanocyte (T: 00:15, 02:00, 03:00, 03:45, 06:30). When there is no contact, fluorescence intensity returns to basal levels (T: 00:00, 01:30, 04:15). A maximum projection of the relevant sections from the confocal stack of the green signal (GFP) from each corresponding time point of the videosequence is shown. *Scale bar* = 20 μm.

The role of tetraspanins in adhesion to the ECM varies among different cell types, and it has been suggested that tetraspanins are not involved in cellular adhesion in most cells (Berditchevski, 2001). In this regard, CD9 did not affect MDA-MB 231 (Sugiura and Berditchevski, 1999), human B cells (Shaw *et al*, 1995) or hepatic cells (Mazzocca *et al*, 2002) adhesion. Accordingly, our data show that tetraspanins slightly modulate the adhesion of melanocytes to collagen. But CD151 increased adhesion to collagen and fibronectin of the erytroleukemia cell line HEL (Fitter *et al*, 1999) but did not affect many other cell lines and human primary cells (Yauch *et al*, 1998; Fitter *et al*, 1999; Sugiura and Berditchevski, 1999; Mazzocca *et al*, 2002). It is remarkable that a defective CD151 protein has been implicated as the cause of epidermolysis bullosa in two patients, suggesting that this tetraspanin could play a role in keratinocyte adhesion (Karamatic Crew *et al*, 2004).

Tetraspanins are important in intercellular contacts (Yáñez-Mó *et al*, 2001; Tarrant *et al*, 2003) since they are clearly accumulated in areas of cell–cell contact in most cellular types, both in homo- and heterotypic interactions (Peñas *et al*, 2000; Longo *et al*, 2001). In fact, the CD151-α3β1 integrin complex has been shown to regulate cadherin-mediated adhesion (Chattopadhyay *et al*, 2003). We have found that melanocytes express CD9 and CD151 in melanocyte–melanocyte contacts and in melanocyte–keratinocyte interactions (unpublished results). Immunofluorescence staining of intercellular homotypic interactions was very conspicuous but not constant, suggesting a dynamic and transient clustering of the tetraspanins at these sites. This effect has not been described on keratinocytes or endothelial cells, where the expression was permanent in homotypic interactions and absent in cellular areas with no cell contact. These data, together with the findings of the time-lapse confocal microscopy with CD9–GFP and CD151—GFP, suggest that tetraspanins are involved in some of the phases of the cell–cell contact, perhaps involved in finding the path of other cells.

Regarding the expression of CD9 and CD151 at the tip of melanocyte dendrites, our findings also suggest a role in the exploration of the external milieu. On cells of neural origin, localized expression of tetraspanins CD9 and CD151 on the tips of the neurites has been implicated in neurite formation (Smith *et al*, 1996; Banerjee *et al*, 1997; Stipp and Hemler, 2000). Our immunofluorescence studies showed that localization of CD9 and CD151 at the tip of melanocyte dendrites was not permanent, and suggested a dynamic clustering of both tetraspanins and β1 integrins. The use of GFP-tagged versions of CD9 and CD151 allowed us to analyze the dynamic clustering of these tetraspanins. Although we cannot exclude that the expression of tetraspanins at the tips of melanocyte dendrites is related to a role in dendrite formation, our data on CD9 and CD151 suggest the involvement of tetraspanins in motility-related structures for the exploration of the environment.

Tetraspanins have been implicated in the motility of many cell types (Berditchevski, 2001), including melanoma cells (Longo *et al*, 2001). The mechanism is still in the process of being elucidated but, as we have previously described in keratinocytes, CD9 and CD151 are present at motility-related structures and not in adhesive contacts to extracellular matrix (Peñas *et al*, 2000). We have found that the anti-CD9 mAb VJ1/10 enhances the motility of melanocytes in migration assays and that it is further enhanced with the anti-β1 integrin VJ1/14. Moreover, CD9 siRNA treatment enhances the migration of these cells, supporting our findings that CD9 expression is involved in the negative regulation of melanocyte migration. In human melanoma cells expressing high levels of CD9, anti-CD9 VJ1/10 slightly inhibited cell migration, although anti-CD9 VJ1/10 and VJ1/20

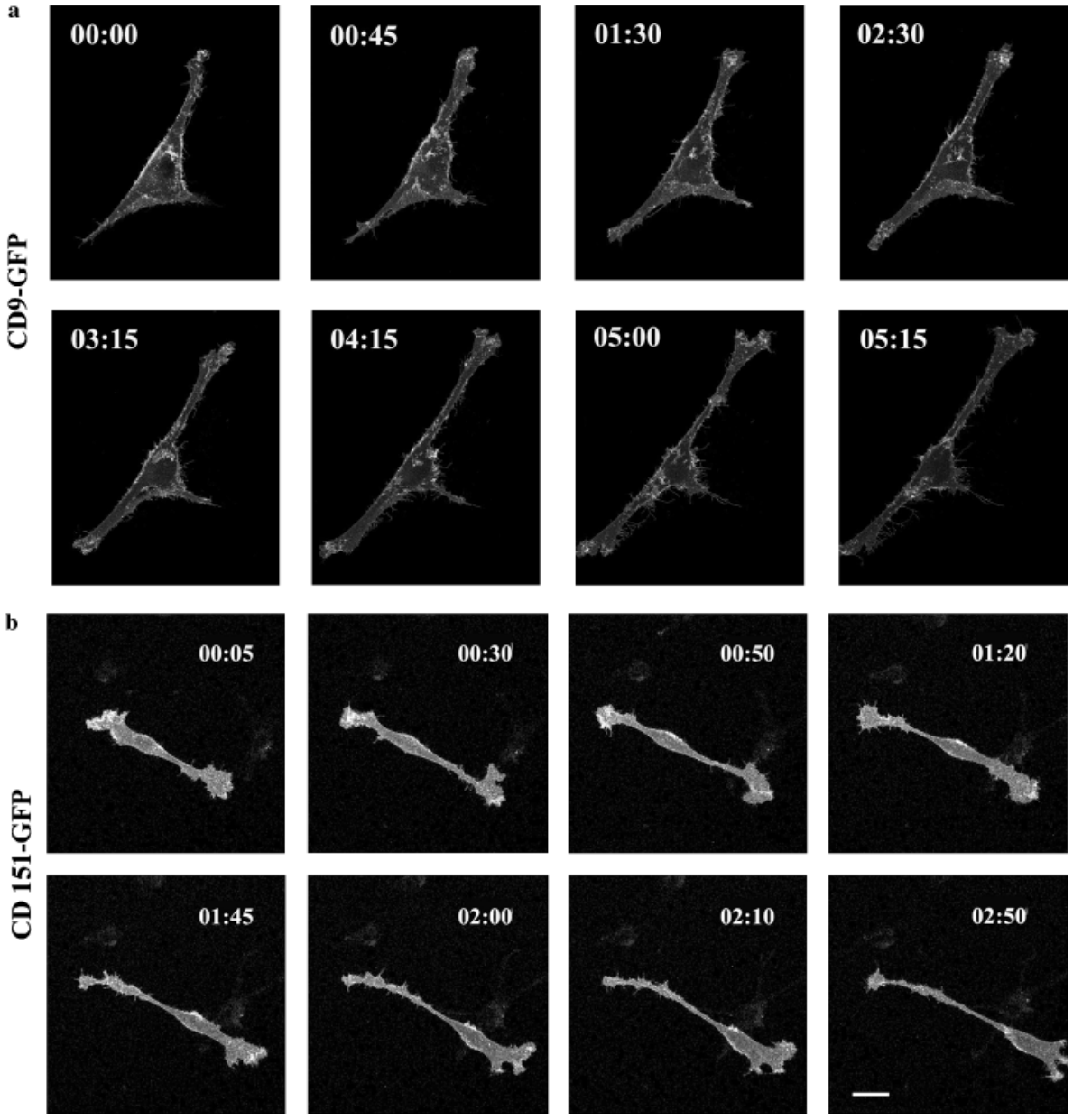

Figure 4
CD9–GFP (green fluorescent protein) and CD151–GFP are expressed on the tips of dendrites of melanocytes during cell migration. Melanocytes transfected with CD9–GFP or CD151–GFP constructs were seeded on coverslips and time-lapse fluorescence confocal microscopy was started after 4 h. Series of optical sections distanced 0.4 μm on the z-axis were acquired every 15 min (CD9) or 5 min (CD151) for a total time of 7 h. (*a*) The micrographs show the dynamic accumulation of CD9–GFP at the tips of the dendrite (T: 00:00, T: 01:30, T: 02:30, T: 04:15) during the exploration of the extracellular environment. A maximum projection of the relevant sections from the confocal stack of the green signal (GFP) from each corresponding time point of the videosequence is shown. *Scale bar* = 20 μm. (*b*) The micrographs show the dynamic accumulation of CD151–GFP at the tip of the dendrite as the melanocyte migrates on the coverslip. CD151 clusters can be found both on the front (00:30, 01:20, 1:45, 2:10) and rear (T: 00:05; 00:30, 00:50) dendrites. A maximum projection of the relevant sections from the confocal stack of the green signal (GFP) from each corresponding time point of the videosequence is shown. *Scale bar* = 20 μm.

presented a strong inhibitory effect of transendothelial migration (Longo *et al*, 2001). Nevertheless, in human and mouse melanoma cells, low CD9 expression has been related to enhanced motility, and transfection of mouse melanoma cells with CD9 lowered their metastatic potential and motility (Ikeyama *et al*, 1993). It is remarkable that mAb anti-CD9 inhibited the migration of human keratinocytes (Peñas *et al*, 2000), endothelial cells (Yáñez-Mó *et al*, 1998), and hepatic stellate cells (Mazzocca *et al*, 2002), whereas it enhanced the migration of Schwann cells (Anton *et al*, 1995) and melanocytes, both of neuroectodermal origin.

On the other hand, the use of anti-CD151 mAb also enhances the migration of melanocytes. In contrast, anti-CD151 mAb inhibited human keratinocyte (Peñas *et al*, 2000), endothelial (Yáñez-Mó *et al*, 1998), neutrophil (Yauch *et al*, 1998), and hepatic stellate cell (Mazzocca *et al*, 2002) migration. Our finding that CD151 siRNA treatment also increased the migration of melanocytes supports that the role

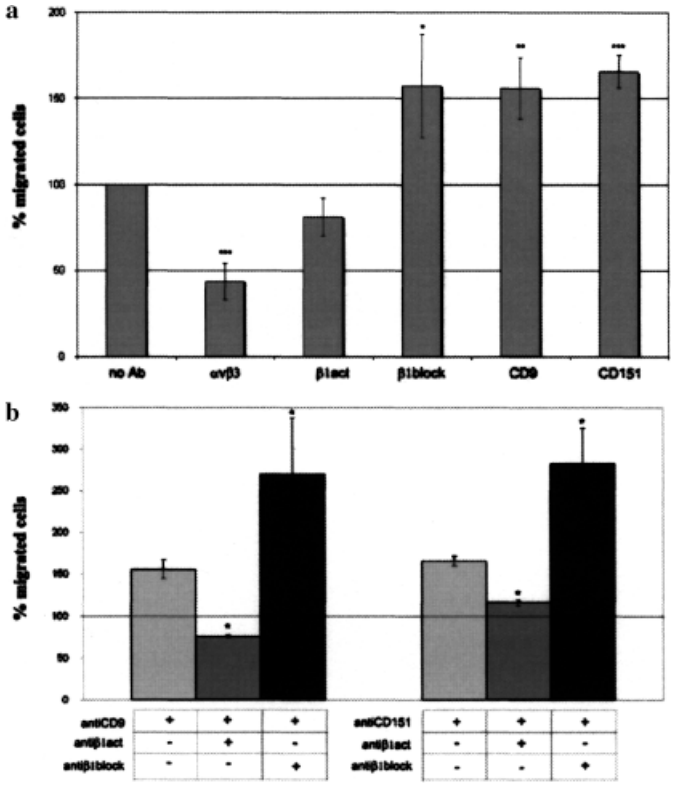

Figure 5
Anti-CD9 and -CD151 monoclonal antibodies (mAb) influence melanocyte motility. Melanocyte migration assays were performed in 8-μm pore Transwell chambers. Fibronectin was added in the lower chamber as a chemoattractant. The cells that crossed the membrane were counted. (*a*) Blocking mAb anti-CD9, anti-CD151, and anti-β1 stimulated the migration of cells, whereas anti-αvβ3 and activating anti-β1 inhibited the migration. Results were normalized using as control mAb-untreated cells as 100%. Results are depicted as the mean ± SEM of seven experiments performed in duplicate. $^{*}p<0.05$, $^{**}p<0.005$, and $^{***}p>0.0005$ with respect to no mAb treatment. (*b*) The same experiment was performed combining the anti-CD9 or anti-CD151 mAb with the activating anti-β1 or the blocking anti-β1 mAb. Results were normalized using the control with no mAb treatment as 100% (*black line*). Results are depicted as the mean ± SEM of two experiments performed in duplicate. $^{*}p<0.05$ with respect to no mAb treatment.

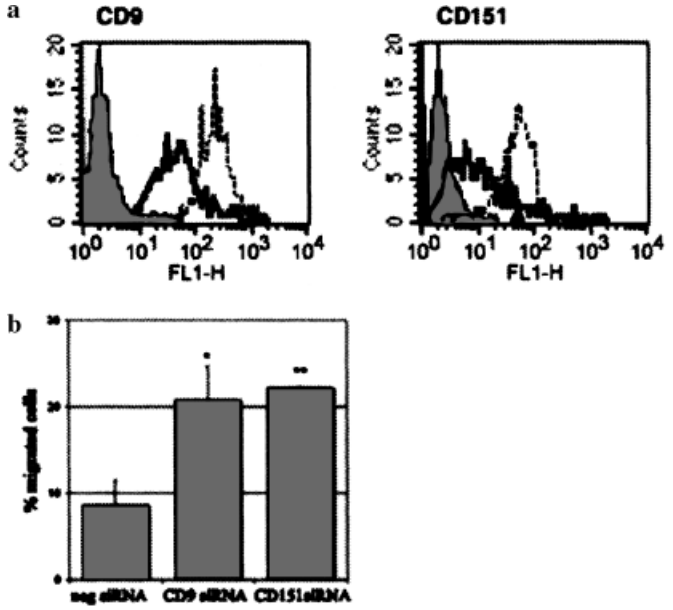

Figure 6
Knocking down of CD9 and CD151 expression using small interference RNA (siRNA) enhances melanocyte motility. (*a*) Melanocytes were transfected with CD9 or CD151 siRNA and negatively selected with anti-CD9 or anti-CD151 magnetic-coated beads, in order to enrich the tetraspanin low-expressing population. We obtained highly interfered melanocyte cells for each tetraspanin. One representative experiment is shown. The mean inhibition was 49% for CD9 and 56% for CD151. Dotted line: negative oligo siRNA-transfected melanocytes, solid line: tetraspanin siRNA-transfected melanocytes, gray area: X-63 used as a negative control of expression. (*b*) The migratory capacity of these transfected melanocytes was evaluated using 8-μm pore Transwell chambers. Fibronectin was added in the lower chamber as a chemoattractant. Negative oligo siRNA-transfected cells were used as control. Results are shown as the mean ± SEM of three experiments performed in triplicate. $^{*}p<0.05$ and $^{**}p<0.005$ with respect to negative oligo siRNA treatment.

of CD151 in melanocyte motility is different from what has been previously described for other cell types.

We have found that tetraspanins modulate migration of melanocytes. Melanocytes express α2β1, α3β1, α5β1, and αvβ3 (Zambruno *et al*, 1993; Hara *et al*, 1994). Tetraspanins have been shown to interact with α2β1 and α3β1 integrins in many cell types, but not with αvβ3 (Berditchevski, 2001). The effect of CD9 and CD151 is partially reverted with TS2/16 (activating anti-β1 integrin mAb), and it is additive to the effect of VJ1/14 (inhibitory anti-β1 integrin mAb).

In conclusion, CD9 and CD151 are dynamically clustered at the tips of dendrites and also in homotypic intercellular contacts of melanocytes. The inhibition of CD9 and CD151 using mAb or siRNA leads to enhanced motility of the cells. Nevertheless, CD9 and CD151 do not seem to be involved in melanocyte adhesion to the extracellular matrix. In keratinocytes, tetraspanins are also involved in motility and are expressed in intercellular contacts (Peñas *et al*, 2000). It is therefore tempting to suggest that the expression of tetraspanins in cell contacts could be negatively regulating melanocyte migration and, therefore, aiding in the process of homogeneous redistribution of melanocytes in the normal epidermis or in the migration of melanocytes to restore the epidermis damaged during wound healing.

Materials and Methods

Cells Neonatal foreskins obtained within 24 h of elective circumcision were used to culture human melanocytes as described previously (Gilchrest *et al*, 1984; Park *et al*, 1993). In brief, epidermal strips were isolated using dispase 1.2 U per mL (Boehringer Mannheim, Mannheim, Germany) for 12 h at 4°C. After incubation of epidermal strips in 0.05% trypsin/0.02% EDTA (Cambrex, Vervies, Belgiun) for 25 min at room temperature, epidermal cells were seeded onto plastic dishes (Corning Incorporated, Corning, New York) at 40,000 cells per cm^2. The culture basal medium consisted in 4:1 Dulbecco's modified Eagle's medium (DMEM) with glutamine and pyruvate (Gibco, Paisley, Scotland, UK) and HAM'S F12 (Gibco) complemented with fetal bovine serum 10%, adenine 18 mM (SIGMA, St Louis, Missouri), hydrocortisone 0.5 μg per mL (SIGMA), apo-transferrin 5 μg per mL (SIGMA, Steinheim, Germany), insulin 5 μg per mL (SIGMA), and choleric toxin 10 ng per mL (SIGMA). In the first passage, a selective tripsinization was performed and the melanocytes were cultured in a melanocyte-specific medium: MCDB153 (SIGMA) 5% fetal bovine serum, bovine

pituitary extract (SIGMA) 30 μg per mL, β-fibroblast growth factor (SIGMA) 0.6 ng per mL, insulin 5 μg per mL, hydrocortisone 0.5 μg per mL, and PHA 8 nM (SIGMA). Cultures were used between the second and fourth passages. Approval was obtained from the Hospital Universitario de la Princesa institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki.

Antibodies MAb used in this study have been described previously: the activatory anti-β1 integrin chain TS2/16 (CD29) (Arroyo *et al*, 1992), the inhibitory anti-β1 integrin VJ1/14 (CD29) (Peñas *et al*, 1998), and the anti-CD151 (LIA1/1 and VJ1/16) (Yáñez-Mó *et al*, 1998). Anti-CD63 (TEA3/18), anti-CD9 (VJ1/20 and VJ1/10), anti-α2 (TEA1/41), and anti-α3 (VJ1/6) were obtained in our laboratory (Peñas *et al*, 2000). JS-81 (anti-CD81) was purchased from BD Biosciences (San Diego, California). The anti-αvβ3 was a kind gift from Dr S. Vilaró (Department of Cell Biology, Universidad de Barcelona, Spain). The monoclonal Ig (IgG1, κ) from the P3X63 myeloma cell line was used as a negative control, and anti-CD45 antibodies (IgG1, IgG2a, and IgG2b) were used as isotype controls (Pulido *et al*, 1988)

Flow cytometry Melanocytes were detached with 0.05% trypsin and 0.02% EDTA, washed, and resuspended in phosphate-buffered saline (PBS). A total of 5×10^5 cells were incubated with the corresponding mAb for 30 min at 4°C. After this incubation, cells were washed in PBS and incubated for 20 min at 4°C with the appropriate fluorescein-isothiocyanate (FITC)-conjugated antibody (anti-mouse Ig from DAKOPATTS, Copenhagen, Denmark or anti-rabbit Ig from Pierce Chemical, Rockford, Illinois). Cells were washed and resuspended in PBS with 10 μg per mL propidium iodide and then analyzed in a FACScan flow cytometer (Becton Dickinson Labware, Lincoln Park, New Jersey). Cell analysis was gated both on forward- and size-scatter intensities and propidium iodide fluorescence to discard dead cells.

Immunofluorescence and laser scanning confocal microscopy Normal human melanocytes were plated in complete medium on glass coverslips, grown, and fixed in 3.7% paraformaldehyde in PBS. Nonspecific binding sites were blocked by incubation with TNB (0.1 M Tris-HCl, 0.15 M NaCl, 0.5% blocking reagent; Boehringer Mannheim GmbH). Coverslips were incubated with the primary antibody for 1 h at 37°C, washed and incubated with Rhodamine-X goat anti-mouse IgG secondary antibody (Molecular Probes, Leiden, The Netherlands) for 45 min. Coverslips were then blocked with mouse serum, incubated with the corresponding biotinylated antibody for 1 h at 37°C, washed, and incubated with Alexa Fluor488-labelled streptavidin (Molecular Probes, Leiden, The Netherlands) for 45 min. Specimens were examined with a Leica TCS-SP confocal laser scanning unit equipped with Ar and He/Ne laser beams and coupled to a Leica DMIRBE inverted epifluorescence microscope (Leica Microsystems, Heidelberg, Germany), using a × 63/1.4 NA oil-immersion objective. A series of optical sections distanced 0.4 μm on the z-axis were obtained.

Migration assay Melanocyte migration assays were performed in 8-μm pore Transwell chambers (Corning Incorporated, Corning, NY). Fibronectin has been described as a stimulus of melanocyte motility (Zambruno *et al*, 1993); therefore, fibronectin 10 μg per mL was added to the lower chamber and incubated for 1 h at 37°C. Melanocytes, resuspended in serum-free medium MCDB153, were then seeded at 15,000 cells/well on the upper chamber. After 30 min, purified monoclonal antibodies were added to the upper chamber where appropriate. After 18 h, migrated cells onto the lower surface of the filter were stained with toluidine blue and counted. Experiments were carried out in duplicate, and four fields of each transwell were counted with a × 40 objective in an Eclipse E400 microscope (Nikon, Tokyo, Japan).

Regarding the migration assay using siRNA-transfected cells, cells were recovered from the lower surface of the filter by trypsinization after the incubation period for migration, and the number of migrated cells was estimated by flow cytometry.

Recombinant DNA constructs, transient transfection, and time-lapse fluorescence confocal microscopy The CD9–GFP fusion protein construct was obtained by polymerase chain reaction amplification of the CD9 cDNA (a kind gift from Dr E. Rubinstein, INSERM, Villejuif Cedex, France) and cloned in pEGFP-N1 Vector (Clontech Laboratories, Palo Alto, California) in *Eco*RI sites of the cloning site, as described elsewhere (Longo *et al*, 2001). The CD151–GFP fusion protein construct was also obtained by polymerase chain reaction amplification of the CD151 cDNA (a kind gift from Dr S. Fujita, Ehime University, School of Medicine, Shigenobu, Japan) and cloned in pEGFP-N1 Vector (Clontech Laboratories) in *Hind*III–*Kpn*1 sites of the cloning site (Barreiro *et al*, 2005).

Primary melanocytes were trypsinized and resuspended in MCDB 153 medium supplemented with 5% FCS, 37.5 mM NaCl, and 20 μg of the vector coding for CD9– or CD151–GFP-tagged proteins. Cells were transfected by electroporation at 975 μF and 200 V in a Gene Pulser II (Bio-Rad, Hercules, California) and were allowed to grow up to 24–48 h after transfection. Then, cells were trypsinized and seeded onto an uncoated 25 mm glass coverslip. Finally, time-lapse videomicroscopy experiments were performed after 4 h of cell adhesion. Samples were maintained at 37°C in a 5% CO_2 atmosphere using an incubation system (La-con GBr Pecon GmbH, Erbach, Germany) coupled to the Leica TCS-SP confocal laser scanning unit. A series of optical sections distanced 0,4 μm on the z-axis were acquired every 5–15 min during a total experimental time of 7 h. Green fluorescence and DIC images were simultaneously obtained using a × 63/1.4 NA oil-immersion objective. Images were processed and assembled into movies using the Leica Confocal Software.

siRNA assay To knock down the expression of CD9 and CD151 selectively, the silencing sequences described previously (Barreiro *et al*, 2005) were used. RNA duplexes corresponding to CD9 and CD151 target sequences, as well as a negative oligonucleotide that does not pair with any human mRNA were produced by Eurogentec (Seraing, Belgium). Oligonucleotides were transfected in primary melanocytes with oligofectamine (Invitrogen, Carlsbad, California) following the manufacturer's instructions. For CD9 interference, melanocytes were transfected on day 0, split on day 2, and retransfected on day 3. For CD151, cells were transfected only once on day 3. Then, cells were trypsinized on day 6 and negatively selected with anti-CD9 or anti-CD151 magnetic-coated beads (Dynabeads M450 Goat anti-Mouse IgG, Dynal Biotech ASA, Oslo, Norway), in order to enrich the tetraspanin low-expressing population. Cells thus selected were counted and seeded for the different experiments.

Statistical analysis Data were compared using the Student's *t* test or one-way analysis of variance.

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Supplementary Material

The following material is available online for this article.

Figure S1. Video S1 (from Fig 3*a*). Primary human melanocytes transiently transfected with CD9-GFP were seeded on a coverslip for 4 h.

Figure S2. Video S1 (from Fig 3*b*). Primary human melanocytes transiently transfected with CD151-GFP were seeded on a coverslip for 4 h.

Figure S3. Video S1 (from Fig 4*a*). Primary human melanocytes transiently transfected with CD9-GFP were seeded on a coverslip for 4 h.

Figure S4. Video S1 (from Fig 4*b*). Primary human melanocytes transiently transfected with CD151-GFP were seeded on a coverslip for 4 h.

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References

Anton ES, Hadjiargyrou M, Patterson PH, Matthew WD: CD9 plays a role in Schwann cell migration *in vitro*. J Neurosci 15:584–595, 1995

Arroyo AG, Sánchez-Mateos P, Campanero MR, Martín-Padura I, Dejana E, Sánchez-Madrid F: Regulation of the VLA integrin-ligand interactions through b1 subunit. J Cell Biol 117:659–670, 1992

Banerjee SA, Hadjiargyrou M, Patterson PH: An antibody to the tetraspan membrane protein CD9 promotes neurite formation in a partially alpha3beta1 integrin-dependent manner. J Neurosci 17:2756–2765, 1997

Barreiro O, Yanez-Mo M, Sala-Valdes M, *et al*: Endothelial tetraspanin microdomains regulate leukocyte firm adhesion during extravasation. Blood 105:2852–2861, 2005

Berditchevski F: Complexes of tetraspanins with integrins: More than meets the eye. J Cell Sci 114:4143–4151, 2001

Berditchevski F, Zutter MM, Hemler ME: Characterization of novel complexes on the cell surface between integrins and proteins with 4 transmembrane domains (TM4 proteins). Mol Biol Cell 7:193–207, 1996

Boucheix C, Rubinstein E: Tetraspanins. Cell Mol Life Sci 58:1189–1205, 2001

Chattopadhyay N, Wang Z, Ashman LK, Brady-Kalnay SM, Kreidberg JA: Alpha3beta1 integrin-CD151, a component of the cadherin–catenin complex, regulates PTPmu expression and cell–cell adhesion. J Cell Biol 163:1351–1362, 2003

Clark EA, Brugge JS: Integrins and signal transduction pathways: The road taken. Science 268:233–239, 1995

Fitter S, Sincock PM, Jolliffe CN, Ashman LK: Transmembrane 4 superfamily protein CD151 (PETA-3) associates with beta 1 and alpha IIb beta 3 integrins in haemopoietic cell lines and modulates cell–cell adhesion. Biochem J 338 (Pt 1):61–70, 1999

Fitzpatrick T, Breathnach A: Das epidermale Melanin-Einheit system. Dermatol Wochenschr 147:481–489, 1963

Gilchrest BA, Vrabel MA, Flynn E, Szabo G: Selective cultivation of human melanocytes from newborn and adult epidermis. J Invest Dermatol 83: 370–376, 1984

Hara M, Yaar M, Tang A, Eller MS, Reenstra W, Gilchrest BA: Role of integrins in melanocyte attachment and dendricity. J Cell Sci 107:2739–2748, 1994

Hemler ME: Specific tetraspanin functions. J Cell Biol 155:1103–1107, 2001

Hemler ME, Mannion BA, Berditchevski F: Association of TM4SF proteins with integrins: Relevance to cancer. Biochim Biophys Acta 1287:67–71, 1996

Hoath SB, Leahy DG: The organization of human epidermis: Functional epidermal units and phi proportionality. J Invest Dermatol 121:1440–1446, 2003

Hsu MY, Meier FE, Nesbit M, Hsu JY, Van Belle P, Elder DE, Herlyn M: E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. Am J Pathol 156:1515–1525, 2000

Ikeyama S, Koyama M, Yamaoko M, Sasada R, Miyake M: Suppression of cell motility and metastasis by transfection with human motility-related protein (MRP-1/CD9) DNA. J Exp Med 177:1231–1237, 1993

Jones PH, Bishop LA, Watt FM: Functional significance of CD9 association with beta 1 integrins in human epidermal keratinocytes. Cell Adhes Commun 4:297–305, 1996

Karamatic Crew V, Burton N, Kagan A, *et al*: CD151, the first member of the tetraspanin (TM4) superfamily detected on erythrocytes, is essential for the correct assembly of human basement membranes in kidney and skin. Blood 104:2217–2223, 2004

Longo N, Yanez-Mo M, Mittelbrunn M, de la Rosa G, Munoz ML, Sanchez-Madrid F, Sanchez-Mateos P: Regulatory role of tetraspanin CD9 in tumor-endothelial cell interaction during transendothelial invasion of melanoma cells. Blood 98:3717–3726, 2001

Maecker HT, Todd SC, Levy S: The tetraspanin superfamily: Molecular facilitators. FASEB J 11:428–442, 1997

Mazzocca A, Carloni V, Sciammetta S, *et al*: Expression of transmembrane 4 superfamily (TM4SF) proteins and their role in hepatic stellate cell motility and wound healing migration. J Hepatol 37:322–330, 2002

Morelli JG, Yohn JJ, Zekman T, Norris DA: Melanocyte movement *in vitro*: Role of matrix proteins and integrin receptors. J Invest Dermatol 101:605–608, 1993

Okochi H, Kato M, Nashiro K, Yoshie O, Miyazono K, Furue M: Expression of tetra-spans transmembrane family (CD9, CD37, CD53, CD63, CD81 and CD82) in normal and neoplastic human keratinocytes: An association of CD9 with alpha 3 beta 1 integrin. Br J Dermatol 137:856–863, 1997

Ota A, Park JS, Jimbow K: Functional regulation of tyrosinase and LAMP gene family of melanogenesis and cell death in immortal murine melanocytes after repeated exposure to ultraviolet B. Br J Dermatol 139:207–215, 1998

Palecek SP, Schmidt CE, Lauffenburger DA, Horwitz AF: Integrin dynamics on the tail region of migrating fibroblasts. J Cell Sci 109:941–952, 1996

Park HY, Russakovsky V, Ohno S, Gilchrest BA: The beta isoform of protein kinase C stimulates human melanogenesis by activating tyrosinase in pigment cells. J Biol Chem 268:11742–11749, 1993

Peñas PF, García-Diez A, Sánchez-Madrid F, Yañez-Mó M: Tetraspanins are localized at motility-related structures and involved in normal human keratinocyte wound healing migration. J Invest Dermatol 114:1126–1135, 2000

Peñas PF, Gomez M, Buezo GF, *et al*: Differential expression of activation epitopes of beta1 integrins in psoriasis and normal skin. J Invest Dermatol 111:19–24, 1998

Pulido R, Cebrián M, Acevedo A, de Landázuri MO, Sánchez-Madrid F: Comparative biochemical and tissue distribution study of four distinct CD45 antigen specificities. J Immunol 140:3851–3857, 1988

Radford KJ, Thorne RF, Hersey P: CD63 associates with transmembrane 4 superfamily members, CD9 and CD81, and with beta 1 integrins in human melanoma. Biochem Biophys Res Commun 222:13–18, 1996

Rubinstein E, Le Naour F, Lagaudriere-Gesbert C, Billard M, Conjeaud H, Boucheix C: CD9, CD63, CD81, and CD82 are components of a surface tetraspan network connected to HLA-DR and VLA integrins. Eur J Immunol 26:2657–2665, 1996

Shaw AR, Domanska A, Mak A, *et al*: Ectopic expression of human and feline CD9 in a human B cell line confers beta 1 integrin-dependent motility on fibronectin and laminin substrates and enhanced tyrosine phosphorylation. J Biol Chem 270:24092–24099, 1995

Sincock PM, Mayrhofer G, Ashman LK: Localization of the transmembrane 4 superfamily (TM4SF) member PETA-3 (CD151) in normal human tissues: Comparison with CD9, CD63, and alpha5beta1 integrin. J Histochem Cytochem 45:515–225, 1997

Smith BE, Bradshaw AD, Choi ES, Rouselle P, Wayner EA, Clegg DO: Human SY5Y neuroblastoma cell interactions with laminin isoforms: Neurite outgrowth on laminin-5 is mediated by integrin alpha 3 beta 1. Cell Adhes Commun 3:451–462, 1996

Stipp CS, Hemler ME: Transmembrane-4-superfamily proteins CD151 and CD81 associate with alpha 3 beta 1 integrin, and selectively contribute to alpha 3 beta 1-dependent neurite outgrowth. J Cell Sci 113:1871–1882, 2000

Stipp CS, Kolesnikova TV, Hemler ME: Functional domains in tetraspanin proteins. Trends Biochem Sci 28:106–112, 2003

Sugiura T, Berditchevski F: Function of alpha3beta1-tetraspanin protein complexes in tumor cell invasion. Evidence for the role of the complexes in production of matrix metalloproteinase 2 (MMP-2). J Cell Biol 146: 1375–1389, 1999

Tarrant JM, Robb L, van Spriel AB, Wright MD: Tetraspanins: Molecular organisers of the leukocyte surface. Trends Immunol 24:610–617, 2003

Watt FM, Hertle MD: Keratinocyte integrins. In: Leight IM, Lane EB, Watt FM (eds). The Keratinocyte Handbook. Cambridge: Cambridge University Press, 1994; p 153–164

Wright MD, Tomlinson MG: The ins and outs of the transmembrane 4 superfamily. Immunol Today 15:588–594, 1994

Yáñez-Mó M, Alfranca A, Cabañas C, *et al*: Regulation of endothelial cell motility by complexes of tetraspan molecules CD81/TAPA-1 and CD151/PETA-3 with alpha3 beta1 integrin localized at endothelial lateral junctions. J Cell Biol 141:791–804, 1998

Yáñez-Mó M, Mittelbrunn M, Sanchez-Madrid F: Tetraspanins and intercellular interactions. Microcirculation 8:153–168, 2001

Yauch RL, Berditchevski F, Harler MB, Reichner J, Hemler ME: Highly stoichiometric, stable, and specific association of integrin alpha3beta1 with CD151 provides a major link to phosphatidylinositol 4- kinase, and may regulate cell migration. Mol Biol Cell 9:2751–2765, 1998

Zambruno G, Marchisio PC, Melchiori A, Bondanza S, Cancedda R, De Luca M: Expression of integrin receptors and their role in adhesion, spreading and migration of normal human melanocytes. J Cell Sci 105:179–190, 1993